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Testing Protocol**

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**Supplemental Assay Method for Titration of Bovine Rotavirus Antibody
(Constant Virus-Varying Serum Method)**

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**Supplemental Assay Method for Titration of Bovine Rotavirus Antibody
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**Supplemental Assay Method for Titration of Bovine Rotavirus Antibody
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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* test for the serum neutralization (SN) antibody titer against bovine rotavirus (BRota). The assay uses a constant amount of virus to test varying dilutions of serum for antibodies against Group A bovine rotavirus in a cell culture system.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Incubator, $36^{\circ} \pm 2^{\circ}\text{C}$, $5\% \pm 1\%$ CO_2 , high humidity

2.1.2 Water bath, $37^{\circ} \pm 1^{\circ}\text{C}$; $56^{\circ} \pm 1^{\circ}\text{C}$

2.1.3 Pipettors, 200- μL and 500- μL and tips

2.1.4 Vortex mixer

2.1.5 Microscope, inverted light

2.1.6 Multichannel pipettor, 50- to 300- μL x 8- or 12-channel

2.1.7 Centrifuge and rotor

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 Cell Cultures

Multiple 96-well disposable plates are seeded (0.2 mL/well) with rhesus monkey kidney (MA-104) cells. Cells must be free of extraneous agents. The cells are seeded at a density that will produce 90-100% confluency after 2 days of incubation.

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2.2.2 Growth Medium

The cells are grown in Minimum Essential Medium (MEM) with 7% fetal bovine serum and additives (**Appendix I**) at a temperature of 35° to 37°C in an incubator containing an atmosphere of 5% carbon dioxide (CO₂) and a relative humidity of 70 to 80%. Growth medium is not changed unless excess acidity occurs or cells are not growing well.

2.2.3 Maintenance Medium

Maintenance medium (**Appendix II**) without serum is used to rinse the cells prior to inoculation. It is also used as a diluent, in the presence of pancreatin, for the serum-virus neutralization assay.

2.2.4 Indicator Virus

The National Veterinary Services Laboratories (NVSL) reference bovine rotaviruses for serotypes 6 (NCDV-Lincoln strain) and 10 (B223 strain) are used as controls for the cell system.

2.2.5 Primary Antibody

When indirect immunofluorescence (IFA) and not cytopathic effect (CPE) is used to titer the virus, serotype- or strain-specific antisera or monoclonal antibodies are used as primary antibody.

2.2.6 Fluorescent Antibody Conjugate

NVSL reference fluorescein isothiocyanate-conjugated immunoglobulin-specific antiserum is used in the IFA assay.

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in cell culture technique and the propagation and maintenance of animal viruses. Personnel shall have an understanding of the immunological basis of SN assays and the principles of aseptic technique.

3.2 Preparation of equipment/instrumentation

3.2.1 On the day of test initiation, set a water bath at 56°± 2°C.

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3.2.2 On the day of test initiation, set a water bath at $37^{\circ}\pm 1^{\circ}\text{C}$.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of MA-104 Plate

Cells are prepared from healthy, confluent MA-104 cells that are maintained by passing every 5 ± 2 days. Two days prior to test initiation, cells are removed from the growth containers by using TV Solution. Using a multichannel pipettor, add 200 μL /well of $10^{5.4}$ to $10^{5.6}$ cells/mL cells suspended in Growth Medium into all wells of a 96-well cell culture plate. Prepare 1 MA-104 plate for the controls and BRota Positive Control Working Dilution. Each additional plate allows testing of 2 Test Serials either pre- or postvaccination. These become the MA-104 Test Plates. Incubate at $36^{\circ}\pm 2^{\circ}\text{C}$ in a CO_2 incubator for 2 days \pm 12 hours. Growth Medium is not changed unless excess acidity occurs or cells are not confluent in 2 days.

3.3.2 Preparation of the BRota Positive Control Working Dilution

1. On the day of test initiation a vial of BRota Positive Control is rapidly thawed in a $36^{\circ}\pm 2^{\circ}\text{C}$ water bath and diluted in Diluent Medium to contain 50-300 50% tissue culture infectious doses (TCID_{50}) per 50 μL . Prior testing of the virus is used to determine the dilution needed to obtain 50 to 300 TCID_{50} for testing. This becomes the BRota Working Dilution.

2. The BRota Working Dilution is back titrated by preparing serial tenfold dilutions (10^{-1} , 10^{-2} , and 10^{-3}) and allowing them to remain at room temperature ($23^{\circ}\pm 2^{\circ}\text{C}$).

a. Place 4.5 mL of Diluent Medium into 3, 17 x 100-mm tubes labeled 10^{-1} - 10^{-3} using a 10-mL serological pipette.

b. Using a 500- μL pipettor, transfer 500 μL of BRota Working Dilution to the 10^{-1} tube; mix by vortexing. Discard pipette tip.

c. Using a new pipette tip, transfer 500 μL from the 10^{-1} labeled tube to the 10^{-2} tube; mix by vortexing. Discard pipette tip.

d. Repeat **Section 3.3.2(2.c)** to the remaining tube, transferring 500 μL from the previous dilution.

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3.4 Preparation of the sample

3.4.1 On day of test initiation or prior to testing (inactivation of pre- and postserums maybe done at time of collection), serums are heat-inactivated at $56^{\circ}\pm 2^{\circ}\text{C}$ for 30 ± 5 minutes.

3.4.2 On day of test initiation the Test Serums (pre- and/or postvaccination serums) are diluted in the Transfer Plate from 1:2 to 1:256 in rows A-H (Appendix I) of a 96-well tissue culture plate labeled as the Test Serum Transfer Plate as described for the PSC and NSC.

4. Performance of the test

4.1 The indicator virus is diluted to contain 100 to 350 TCID₅₀ per 0.2 mL, using maintenance media containing a previously titrated amount of pancreatin, the maximum that the MA-104 cells will tolerate. This dilution is determined by previous titrations and is designated the “stock virus”. The dilution factor is calculated by dividing the titer of the indicator virus by the desired titer of stock virus.

4.2 The serum to be assayed is heat-inactivated at 56°C for 30 minutes. Serial twofold dilutions are made in sterile tubes with diluent containing pancreatin and mixed using a Vortex or similar type of mixer.

4.3 An equal volume of stock virus and of each serum dilution are added together, mixed, and allowed to incubate at 37°C for 60 minutes. The mixing of equal volumes of serum and virus results in a further twofold dilution of serum.

4.4 Cells that have been seeded in 96-well plates 2 to 3 days previously are inverted and the growth medium removed by gentle shaking and tamping on sterile gauze. The cells are rinsed with 0.2 mL of maintenance medium per well, the medium again decanted from the plate, and the cells refed with 0.2 mL of maintenance media which remains on the plate for 1 hour at 37°C .

4.5 The final rinse is removed from the cells, as above. Each well is inoculated with 0.2 mL per well of each stock virus-serum dilution mixture, at a minimum of 4 wells per dilution. A minimum of 8 wells remain uninoculated with virus, to serve as negative cell controls; they receive 0.2 mL per well of only the pancreatin-containing diluent.

4.6 The stock virus is back-titrated by preparing serial tenfold dilutions (10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}) in diluent containing pancreatin. These are then mixed with an equal volume of diluent containing pancreatin and at least 4 wells per dilution are inoculated with 0.2 mL of the mixture.

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4.7 The plates are incubated at 37°C, in an atmosphere of 5.0% CO₂ and high humidity, for 5 days. After 5 days, the cells can be examined for CPE typical of bovine rotavirus and the 50% endpoints calculated. The TCID₅₀ of the stock virus is calculated by the Spearman-Kärber method; its titer must be between 50 and 350 TCID₅₀ per 0.2 mL for a test to be valid. The cells in the negative control wells must remain normal.

4.8 Certain strains of bovine rotavirus may not exhibit pronounced CPE, thus an IFA assay may be necessary to determine their titer.

4.8.1 After the medium is decanted, the cells are gently rinsed in phosphate buffered saline (PBS), then in deionized water. The cells are fixed in a solution of 80% acetone-20% deionized water at 4°C for 15 minutes. The acetone is discarded and the plates air-dried.

4.8.2 The wells are covered with 0.05 mL per well of previously-titrated dilution of specific primary antibody and held in a high humidity, 37°C incubator for 30 minutes. Excess primary antibody is washed from the plates by two gentle PBS rinses and one deionized water rinse. The plates are shaken gently and lightly touched to an absorbent towel to remove excess moisture.

4.8.3 While still moist, wells are covered with 0.05 mL per well of species-specific, conjugated anti-immunoglobulin antiserum. Again, the plates are incubated at 37°C for 30 minutes. Washing is repeated, as in **Section 4.8.2**. The plates are air-dried, face-down.

4.8.4 The cell monolayers are examined by fluorescent microscopy using a Ploem illuminator and blue light (Xenon lamp). Any cells showing immunofluorescence characteristic of bovine rotavirus are considered positive and the 50% endpoints calculated. The TCID₅₀ of the stock virus is calculated using the Spearman-Kärber method; its titer must be between 50 and 350 TCID₅₀ per 0.2 mL for a test to be valid. Also, the non-inoculated wells must be negative for immunofluorescence.

5. Interpretation of the test results

5.1 For a valid assay the following criteria must be met; otherwise the test is considered a **No Test** and is repeated without prejudice.

5.1.1 The NSC must have an SN titer of <1:2.

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5.1.2 The SN titer of the PSC should vary by no more than twofold from its mean titer as established from a minimum of 10 previously determined SN titrations.

5.1.3 The uninoculated cell controls can not exhibit any CPE or cloudy media that would indicate contamination.

5.1.4 The BRota Back Titration titer must be between 50 to 350 TCID₅₀/25 µL.

5.2 For a **SATISFACTORY TEST**, the postvaccination titers shall meet the requirements as stated in an Animal and Plant Inspection Service (APHIS) filed Outline of Production

5.3 If postvaccination titers are less than the requirements in an APHIS filed Outline of Production, the sera will be retested (first retest).

5.3.1 If the titers of the Test Serum from the first valid retest are less than the required titers in an APHIS filed outline of Production, the serial is **UNSATISFACTORY**.

5.3.2 If the titers of the Test Serum from the first valid retest are greater than or equal to the titer in an APHIS filed Outline of Production, the serum will be retested (second retest).

5.3.3 If the titers of the Test Serum from the second valid retest are greater than or equal to the titer in an APHIS filed Outline of Production, the serial is **SATISFACTORY**.

5.3.4 If the titers of the Test Serum from the second valid retest are less than the titers in an APHIS filed Outline of Production, the serial is **UNSATISFACTORY**.

6. Report of test results

6.1 Results are reported as SN titers.

6.2 Record all test results on the test record.

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7. References

7.1 Code of Federal Regulations, Title 9, Part 113.6, U.S. Government Printing Office, Washington, DC, 2007.

7.2 Finney, DJ 1978. *Statistical method in biological assay*. Griffin, London. 3rd edition, pg 508.

7.3 Cottral, GE, (Ed.) 1978, *Manual of standard methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, NY, pg. 731.

8. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes impacting the test were made from the previous protocol, the following changes were made to the document:

- Contacts, Joseph Hermann and Peg Patterson, have been added to the document.

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**Appendix I
Growth Medium**

MEM (Eagle) with Earles salts	1.0 packet
Deionized water q.s.	1.0 liter
Sodium bicarbonate	2.2 gram
Gentamicin sulfate	50.0 mg
Penicillin	25,000 U
Streptomycin	100.0 mg
Heat inactivated or irradiated fetal bovine serum	70.0 mL
200 mM L-Glutamine (100X)	292.0 mg
0.22 micron filtration	

**Appendix II
Maintenance Medium**

MEM (Eagle) with Earles salts	1.0 packet
Deionized water q.s.	1.0 liter
Sodium bicarbonate	2.2 gram
Gentamicin sulfate	50.0 mg
Amphotericin B	5.0 mg
Penicillin	100,000 U
Streptomycin	100.0 mg
200 mM L-Glutamine (100X)	292.0 mg
0.22 micron filtration	